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(54) **A method and reagent combination for the identification of microorganisms and the use of sandwich hybridization of nucleic acids therefor.**

(57) Sandwich hybridization of nucleic acids can be used for the identification of microorganisms or groups of microorganisms present in a sample. The technique can be used to identify, from a single sample containing nucleic acids of the microorganisms or groups of microorganisms to be identified, after first rendering the said nucleic acids single-stranded, all the microorganisms or groups of microorganisms by adding to the sample a pair of nucleic acid reagents for each microorganism or group of microorganisms to be identified, of which pair one nucleic acid reagent is attached to a solid carrier in the single-stranded form and the other contains a completely different nucleic acid fragment from the same microorganism or group of microorganisms, which fragment is labelled with a marker. The nucleic acid fragments of a pair hybridize to a complementary single-stranded nucleic acid from the sample and the hybrid thus formed on the solid carrier is labelled with the marker. Because the labelled nucleic acid reagent does not hybridize directly with the reagent attached to the solid carrier, only those carriers having attached nucleic acid reagents corresponding to nucleic acids in the sample become labelled and the presence of such carrier-bound labels can be measured using established methods.

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A method and reagent combination for the identification of microorganisms and the use of sandwich hybridization of nucleic acids therefor

The present invention relates to the use of sandwich hybridization of nucleic acids for the identification of microorganisms, and to a method and a combination of reagents for this purpose.

In traditional microorganism detection and identification techniques the presence of a microorganism in a given sample is demonstrated by isolation of the microbe in question. After enrichment cultivations the microorganism is identified either on the basis of its biochemical properties or with the use of immunological methods. These techniques require the microorganism in the sample to be viable. Identification by isolation is moreover a laborious method, which in the case of viruses may require 4 - 6 weeks.

The purpose of this invention is to provide a method in which the presence of a microorganism in a sample is demonstrated by the identification of its genetic material, the nucleic acid, with the aid of the sensitive and specific nucleic acid hybridization technique. In itself, nucleic acid hybridization is a known method for investigating the identity of nucleic acids. Complementary nucleic acid strands have the ability to form a tight double-stranded structure according to the rules of base pairing, and the resulting hybrid can be separated from the residual single-stranded nucleic acid.

Some methods based on the identification of nucleic acid(s) have already been applied to microorganism identification. Enterotoxigenic Escherichia coli have been identified from faecal samples by colony hybridization using the gene for toxin production as a probe. Positive hybridization is demonstrated by autoradiography (Moseley,

S.L. et al., J. Infect. Dis. (1980) 142, 892 - 898). Colony hybridization is based on the method originally developed by Grünstein and Hogness (Proc. Natl. Acad. Sci. USA (1975) 72, 3961 - 3965). Hybridization has also been used as a method to distinguish between Herpes simplex virus type 1 and type 2 (Brautigam, A.R. et al. J. Clin. Microbiol (1980) 12, 226-234), however not in rapid diagnostics but in typing of the virus after enrichment cultivation. In this method the double-stranded hybrid is separated by affinity chromatography from the fraction of nucleic acids remaining single-stranded in the solution.

It was recently published that DNA from cells infected with Epstein-Barr virus (the sample) had been fixed onto the filters after appropriate pretreatment. This nucleic acid was identified by hybridizing the filters with the radioactive probe and positive hybridization was detected by autoradiography (Brandsma, I. and Miller, K. (1980) Proc. Natl. Acad. Sci. USA 77, 6851 - 6855).

The invention described herein is based on the sandwich hybridization technique (Dunn, A.R. and Hassell, J.A. (1977) Cell 12, 23 - 36), which simplifies the handling of the sample and the detection of the hybrid. For this reason the technique is particularly suitable for diagnostic use.

Accordingly, the invention provides for the use of the technique of sandwich hybridization of nucleic acids, said technique comprising contacting single-stranded nucleic acid from a microorganism with a pair of different nucleic acid reagents, both reagents of the pair being single-stranded and complementary with the microorganism-derived nucleic acid and one of the pair being a nucleic acid fragment attached to a solid carrier whilst the other is a nucleic acid fragment labelled with a marker, whereby a labelled hybrid is formed attached to the solid carrier,

for the identification of a microorganism or group of microorganisms present in a sample, the pair of nucleic acid reagents being selected according to the microorganism or group of microorganisms expected to be present in the sample and the correctness of the identification being tested by detection of the extent of formation of a labelled hybrid attached to the solid carrier.

By means of this invention all the microorganisms or groups of microorganisms present can be identified from one and the same sample, which contains denaturated single-stranded nucleic acid strands from the microorganisms, without sample division. A pair of the different nucleic acid reagents is required for each microorganism or group of microorganisms to be identified. The pair of reagents are two separate nucleic acid fragments derived from the genome of the microorganism to be identified, which have no sequences in common but preferably are situated close to each other in the genome. The reagents can be prepared directly from the microbial genomes or by using the established recombinant DNA techniques. Of the two nucleic acid fragments, one is fixed to a solid carrier, preferably a nitrocellulose filter, after being denaturated and the other, also in single-stranded form, is labelled with a suitable marker label. When these nucleic acid reagents, two different reagents for each microorganism or group of microorganisms to be identified, are placed in contact with the single-stranded nucleic acids to be identified in the sample, the nucleic acids in the sample anneal to the complementary nucleic acid fragments on the solid carrier. The hybrids thus formed on the carrier become labelled by annealing to the labelled complementary nucleic acid fragments. The labelled nucleic acid fragments do not hybridize directly with the nucleic acid fragments attached to the solid carrier, but hybridize only with the correct single stranded nucleic acids orginating from the sample. Thus only those carriers to which the complemen-

tary nucleic acids from the sample have hybridized can become labelled. These carriers are easily washed and the presence of the label measured by established methods.

The invention can in principle be used for the identification of all organisms containing either DNA or RNA, such as viruses, bacteria, fungi and yeasts. It has the specific advantage of permitting identification of all the bacteria and viruses possibly in question at the same time and from the same sample, regardless of whether the microorganisms contain DNA or RNA. By suitable combination of pairs of reagents it is possible to develop "kits" such that each microorganism to be identified has its own specific pair of reagents, one attached to a solid carrier and the other labelled. All the solid carriers included in the reagent combinations can be added to the sample simultaneously, along with the labelled nucleic acid reagents. When hybridization has taken place, the solid carriers are washed and their labelling is measured. The only carrier or carriers to become labelled are those which contain sequences complementary to the microbial genome in the investigated sample.

The invention can be used, for example, in medical microbiology, veterinary microbiology, food hygiene investigations and microbiological investigation of plant diseases. Suitable sample materials are for example all animal- and plant tissue homogenates, and human and animal secretions such as blood, faeces and nasal- and urethral mucous. The procedure can be made sufficiently sensitive to detect the microorganism levels normally present in clinical samples. Preliminary enrichment of the microorganism(s) present in the sample by cultivation is of course possible before the identification test and in some cases would be essential. The invention is also suitable for the investigation of samples from which the microorganism can no longer be cultivated but which contain considerable amounts of microbial debris (eg. after the

commencement of antibiotic treatment), or when cultivation of the microorganism is particularly laborious and difficult (eg. anaerobic bacteria, which are present in large numbers in suppurative samples in the case of infections caused by anaerobes).

The invention may be utilized, for example, to provide reagent combinations for detection and identification of the following groups of pathogenic microorganisms by appropriate formation of the necessary pairs of nucleic acid reagents. Of course reagent combinations need not contain pairs of reagents specific to each one of the organisms or groups of organisms named under the various individual headings.

Respiratory infections:

a) Bacteria: β-haemolytic streptococci (A-group), Haemophilus influenzae, Pneumococci, Mycoplasma pneumoniae, mycobacteria

b) Viruses: Influenza A, Influenza B, Parainfluenza 1-3 Respiratory syncytial virus, adenoviruses, corona viruses, rhinoviruses

Diarrhoeas:

a) Bacteria: salmonellae, shigellae, Yersinia enterocolitica, enterotoxigenic, E. coli, Clostridium difficile, campylobacteria

b) Viruses: rotaviruses, parvoviruses, adenoviruses, enteroviruses

Venereal diseases:

a) Bacteria: Neisseria gonorrhoeae, Treponema pallidum, Chlamydia trachomatis

b) Viruses: Herpes simplex-virus

c) Yeasts: Candida albicans

d) Protozoa: Trichomonas vaginalis

Sepsis:

a) Bacteria: β-haemolytic streptococci (A-group), pneumococci, enterobacteria as a single group

Food hygiene:

a) Bacteria: salmonella and Clostridium perfringens

Depending on the choice of reagents the specificity of the test can be limited to a single microorganism or a small group of microorganisms (e.g. salmonella bacteria) or to a wider group (e.g. enterobacteriaceae) by choosing identifying reagents from the area of a common gene.

The nucleic acid reagents required in the sandwich hybridization technique described in this invention may be produced by recombinant DNA technology or directly from the genome.

By way of illustration the reagent production and test procedure for example 1 will now be described, but it will be appreciated by those skilled in the art that appropriate modifications may be made without departing from the basic principles of the invention, and that these methods and procedures can be adapted for the production of other pairs of nucleic acid reagents.

Reagents

Adenovirus type 2 (strain deposited at KTL, i.e. the Public Health laboratory, Helsinki) was cultivated and purified

and DNA was isolated (Petterson, U. and Sambrook, J. (1973) J. Mol. Biol. 73, 125-130) (referred to hereafter as Ad_2-DNA). The DNA was digested with BamHI-restriction enzyme (BRL, i.e. Bethesda Research Laboratories), which cuts the DNA into four reproducible fragments. Of these four fragments two were inserted to the BamHI-site of the vector plasmid pBR322 (BRL) with the aid of T4-ligase (BRL). (The fragments were not separated before ligation, but the insert added to the plasmid was in each case identified only after cloning). Subsequently the bacterial host (E. coli HB101 (K12) gal^-, pro^-, leu^-, hrs^-, hrm^-, recA, str^r, F^-) (obtained from KTL) was transformed with the plasmid DNA also containing recombinant plasmids, i.e. molecules which had accepted fragments of the adenovirus-DNA (Cohen, S.N. et al. (1972) Proc. Natl. Acad. Sci. USA 69, 2110-2114). Among the transformed bacterial clones those that most probably contained the recombinant plasmid were chosen. Ampicillin-resistance and tetracycline-resistance are transferred to the bacterium by the pBR322-plasmid (Bolivar F. et al. (1977) Gene 2, 95-113). Bacteria containing the recombinant plasmid are, however, sensitive to tetracycline, because the BamHI-restriction site is within the tetracycline gene and the insertion of foreign DNA into this region destroys the gene. The insertion of the plasmid was characterized after plasmid enrichment by determining the size of the restriction fragments after BamHI digestion using agarose gel electrophoresis. The adjacent BamHI D- and C-fragments of the Ad_2-DNA (c.f. gene map) were chosen as reagents (Söderlund, H. et al. (1976) Cell 7, 585-593). The desired recombinant plasmids, Ad_2C-pBR322, KTL no. E231 and Ad_2D-pBR322, KTL no. EH230, were cultivated and purified as has been described in the literature (Clewell, D.B. and Helinski, D.R. (1969) Proc. Natl. Acad. Sci. USA 62, 1159-1166).

The recombinant plasmid Ad_2D-pBR322 was used as the carrier-bound reagent. It is not necessary for the present inven-

tion to remove the plasmid sequences, because the sample does not contain pBR322-sequences. However, for radioactive labelling the insert nucleic acid was separated from pBR322-DNA after BamHI-digestion with the aid of agarose gel electrophoresis. The C-fragment was isolated from LGT-agarose (Marine Colloids, Inc.) by phenol extraction or electro-elution (Wieslander, L. (1979) Anal. Biochem. 98, 305-309) and concentrated by ethanol precipitation.

It is particularly desirable to subclone the nucleic acid fragment chosen for labelling in a separate vector, in order to avoid the hybridization background resulting from the direct hybridization with the carrier-bound reagent of the residual plasmid sequences, contaminating the labelled nucleic acid reagent. The single-stranded DNA-phage M13 mp7 (BRL), to which DNA fragments obtained by BamHI digestion can easily be transferred, may be used as an optimal vector (Messing, J. et al. (1981) Nucleic Acids Res. 9, 309-323).

Attachment of DNA to the solid carrier (filter)

The recombinant plasmid Ad_2D-pBR322 was denatured to a single stranded form and nicked randomly at several sites by treatment with 0.2 N NaOH (5 min. $100^{o}C$), whereafter the DNA was chilled and, immediately prior to transference to the filter, neutralized and pipetted to the transfer solution, 4 x SSC medium on ice (SSC = 0.15 M NaCl, 0.015 M Na-citrate). The filters (Schleicher and Schüll BA85 nitrocellulose) were thoroughly wetted in 4 x SSC solution (about 2 h) before the application of DNA. The DNA was attached to the filter in a dilute solution (0.5-1.0 µg/ml) by sucking the solution through the filter in a weak vacuum. The filter is capable of absorbing DNA up to about 180 $\mu g/cm^2$ (Kafatos, F.C. et al. (1979) Nucleic Acids Res. 7, 1541-1552). We have used DNA-concentrations of between 0.5 µg DNA/2.5 cm diameter of filter and 1.0 µg DNA/0.7 cm diameter of filter.

After DNA-filtration the filters are washed in 4 x SSC, dried at room temperature and finally baked in a vacuum oven at 80°C for 2 h, after which the DNA on the filters remains stable and the filters can be stored for long periods at room temperature (Southern, E.M. (1975) J. Mol. Biol. 98, 503-517).

Labelling of the radioactive nucleic acid fragment

The radioactive label used was the ^{125}I-isotope. This isotope can be detected using γ-counters, which are available in most large laboratory units. The half-life of the isotope is 60 days, for which reason the utilization period of ^{125}I-labelled reagents is about 4 months.

"Nick-translation" labelling

The principle of this method is to displace one of the nucleotides in the nucleic acid with a radioactive one, upon which the whole DNA molecule becomes labelled. This is carried out according to the method published by Rigby, P.W.J. et al. (J. Mol Biol. (1977) 113, 237-251). In the reaction the DNA becomes labelled when the solution contains ^{125}I-labelled deoxynucleoside triphosphate as substrate, in this case ^{125}I-dCTP (Radiochemical Centre, Amersham: >1500 Ci/mmol). Under optimal conditions a specific activity of 10^9 cpm/μg DNA can be obtained. The labelled DNA is purified from nucleotides remaining in the reaction mixture by simple gel filtration, eg. using BioGel P30 (BioRad).

Other labelling methods

The single-stranded nucleic acid reagent produced in M13 mp7-phage is labelled by chemical iodination, in which the reactive ^{125}I is added covalently to the nucleic acid (Commerford, S.L. (1971) Biochemistry 10, 1993-2000,

Orosz, J.M. and Wetmur, J.G. (1974) Biochemistry 13, 5467-5473). Alternatively, the nucleic acid can be made radioactive by end-labelling with radioactive nucleotides by means of terminal transferase (Roychoudhury, R. and Wu, R. (1980) Meth. Enzymol. 65, 43-62).

The reagent preparation described above relates to microbes of which the genetic material is in the form of DNA. In the case of RNA viruses the cloning of genome fragments can take place after a DNA copy (cDNA) of the virus RNA has been made with the aid of reverse transcriptase, followed by DNA-polymerase to copy the second DNA strand, thereafter the DNA may be cloned as described above (Salser, W. (1979) in Genetic Engineering, Ed. A.M. Chakrabarty, CRC Press, pp. 53-81).

The most suitable cloning method should be chosen depending on the microbe used. The hosts as well as the vectors can vary. Possibilities include the λ-phage as vector, other plasmids, cosmids, and cloning, eg. in Bacillus subtilis bacteria. (Recombinant DNA, Benchmarck Papers in Microbiology, Vol. 15, Eds. K.J. Denniston and L.W. Enqvist, Dowden, Hutchinson and Ross, Inc. (1981); Ish-Horowicz, D. and Burke, J.F. (1981) Nucleic Acids Res. 9, 2989-2998).

Performance of the test

Sample treatment

The microbial nucleic acid to be investigated must be released from within the microbe itself and also from the infected cells, after which it must be denatured to the single-stranded form. Virus genomes can be liberated by treating the sample material with 1 % sodium dodecylsulphate (SDS) and destroying the proteins protecting the genome by proteinase K-treatment (1 mg/ml, 37°C, 60 min). Bacterial

samples must in addition be broken down using lysozyme- and EDTA-treatment.

If the sample contains large quantities of viscous high-molecular weight cellular-DNA, this must be sheared at a few sites in order to reduce its viscosity, eg. by sonication or by passing the sample a few times through a fine needle.

Hybridization

Hybridization takes place eg. in 50 % formamide (deionized, stored at -20^{o}C), in 4 x SSC, Denhardt solution (Denhardt, D.T. 1966) Biochem. Biophys. Res. Commun. 23, 641-646) containing 1 % SDS and 0.5 mg/ml DNA (salmon sperm or calf thymus) at 37^{o}C and usually overnight for 16-20 hours. The filters chosen for the test are incubated in a suitable vessel, to which the hybridization mixture is added and the hybridization is started. The hybridization mixture contains (a) the pretreated sample to which is added the radioactive nucleic acid reagent(s), which are denaturated together by boiling for 5 minutes followed by quick cooling at 0°C; (b) concentrated formamide-, SSC- and Denhardt-solutions, which are pipetted to the denatured and cooled nucleic acid mixture (a). After mixing, the hybridization mixture is pipetted to the filters in the hybridization vessel. After hybridization the filters are carefully washed and counted individually in the γ-counter.

The invention will now be further described, by way of illustration only, with the aid of some practical Examples.

Example 1

Detection of adenovirus by the sandwich hydridization method (Table 1)

The details of the test are clarified in the text to Table 1. The sandwich hybridization method can detect

virus-DNA from a solution, but the viral genome can equally well be detected from infected cells.

The hybridization background is measured in a tube containing only the filter and the labelled nucleic acid reagent, without the sample. The background results from the pBR322 sequences occurring in the labelled nucleic acid reagent. These sequences hybridize directly with the filter without the sample mediating it. The filters containing calf thymus and no DNA are used in the test as controls, indicating on the one hand the specificity of hybridization and on the other the level of the nonspecific background arising eg. from insufficient washing.

In the following tables the background due to the reagents has been subtracted from the cpm-values hybridized to the filters.

Table 1

Adenovirus test

Sample	Filters (cpm)		
	Adeno 1)	Calf thymus 2)	Blank 3)
Adenovirus type 2-DNA (BRL) (500 ng)	9000	49	-
HeLa-cells (6 x 10^5) infected with adenovirus	8200	-	-

Filters:

1) Ad_2D-pBR322-plasmid, 2 μg
2) Calf thymus DNA 1 μg (Boehringer Mannheim)
3) Blank (no DNA)

Labelled nucleic acid reagent:

Ad_2-BamHI C-fragment, purified, specific activity 90 x 10^6 cpm/µg (200000 cpm ^{125}I/reaction)

Hybridization:

50 % formamide, 4 x SSC

Denhardt solution, containing 0,5 mg/ml salmon sperm DNA and 1 % SDS, 37°C, 16 h

Washing:

0,1 x SSC, room temperature, 40 min

Samples:

Adenovirus type 2 DNA (BRL)

Infection with type 2 adenovirus took place in HeLa-cells. The cells were then disrupted by treatment with 1 % SDS, followed by digestion with 1 mg/ml proteinase-K-enzyme (Sigma) for 30 min 37°C. Before denaturation the sample was passed through a fine needle. The values appearing in the table have been corrected by subtraction of the reagent background, obtained by carrying out a similar hybridization but without sample.

Example 2

Detection of an RNA-virus with the aid of sandwich hydridization (Table 2)

The model RNA-virus used was the Semliki Forest virus (prototype strain, obtained from the London School of Hygiene and Tropical Medicine), of which the genome is single-stranded RNA. Using the virus genome as a template cDNA was produced, which was cloned into the pstI site of pBR322 plasmid as described by Garoff et al. (Proc. Natl. Acad. Sci. (1980) USA 77 6376-6380). The recombinant plasmid thus obtained is pKTH312 KTL no. EH 232. The insert of this plasmid originating from the virus genome is about

1400 nucleotides long and is from the structural protein area, approximately from nucleotide 200 to nucleotide 1600 when numbering is started from the beginning of the structural genes (Garoff, H. et al. 1980). For the production of reagent the whole recombinant plasmid pKTH312 was linearized with EcoRI restriction enzyme (BRL). The sequence originating from the Semliki Forest virus does not contain recognition sites for the EcoRI-enzyme), and the linearized plasmid was cut into two fragments using XhoI-enzyme (BRL). The restriction site of the latter was located within the Semliki Forest virus sequence. The larger EcoRI-XhoI-fragment A (about 3900 base pairs) was attached to the filter and the smaller fragment B (about 1850 base pairs) was labelled with ^{125}I using the nick translation technique.

Both free Semliki Forest virus and virus-infected cells were used as samples in this test. In both cases the virus-specific nucleic acids of the sample were composed entirely of RNA.

Table 2

Detection of Semliki Forest virus with the aid of the sandwich hybridization method.

Sample	Filters (cpm)		
	Semliki Forest virus 1)	Calf thymus 2)	Blank 3)
Semliki Forest virus 30 μg	3340	-	33
Cells infected with Semliki Forest virus (5×10^5)	2698	8	10
Non-infected cells	10	5	8

Filters:

1) EcoRI-XhoI-fragment A (1.2 μg) of the pKTH312 plasmid
2) Calf thymus DNA 1 μg
3) Blank (no DNA)

Labelled nucleic acid reagents:

EcoRI-XhoI-fragment B of the plasmid pKTH312, specific activity 90 x 10^6 cpm/μg DNA (200000 cpm ^{125}I/reaction).

Hybridization:

As described in Table 1

Washing:

As described in Table 1

Samples:

Semliki Forest virus (30 μg) was disrupted with SDS before the test. The infected cells were handled as described in Table 1. The infection with Semliki Forest virus was carried out in BHK-21 cells.

The values given in the table have been corrected for reagent background, obtained from a similar hybridization without sample.

Example 3

A virus sample in which the viral messenger RNA is detected with the aid of the sandwich hybridization method (Table 3)

The filter hybridization reagents were produced from SV40-virus DNA (BRL) by cutting the DNA into two parts using PstI-enzyme (Boehringer Mannheim) as described by Lebowitz and Weissman (Curr. Topics in Microbiol. Immunol. 87, 43-172) and the fragments were isolated and purified by agarose gel electrophoresis. Fragment A (4000 base pairs) was radioactively labelled with ^{125}I by nick translation and

fragment B (1220 base pairs) was attached to the filter.

The DNA fragments were chosen so that each contained areas coding for both early and late messengers. Thus fragment B contains about 700 bases from the structural protein gene VP1 and over 600 bases from the gene for early messengers. Because the DNA of SV40 virus is in itself a covalently closed ring, it cannot be detected by the test before linearization. Therefore, when infected cells are used as the sample it is possible to test how well the method is adaptable to the detection of RNA copies of the viral genome. As can be seen from the results in Table 3, the test is excellently suited to the investigation of infected cells. The table also demonstrates that the same reagents can be used to investigate both the viral DNA and mRNA made from it.

Table 3

Detection of SV40-virus by the sandwich hybridization technique

Sample	Filters (cpm)		
	SV40 1)	Calf thymus 2)	Blank 3)
Test 1			
SV40 viral DNA (50 ng) (linearized)	20061	159	104
No sample	-	-	-
Test 2			
CV1-cells infected with SV40-virus 40 h after infection (10^6 cells)	30814	294	580
Non-infected cells	-	-	-

Filters:

1) The shorter fragment PstI B (0.2 μg) of the circular SV40-virus DNA digested with PstI-restriction enzyme
2) Calf thymus DNA 1 μg
3) Blank (no DNA)

Labelled nucleic acid reagent:

The longer PstI A-fragment of the SV40-virus DNA, specific activity 28×10^6 cpm/μg DNA (200000 cpm ^{125}I/reaction)

Hybridization:

As described in Table 1
The hybridization time is 40 h

Washing:

As described in Table 1

Samples:

SV40-virus DNA (BRL) was linearized with EcoRI restriction enzyme (BRL). CV1-cells (Biomedical Centre, Upsala University) were infected with SV40-virus (obtained from Janice Y. Chou and Robert G. Martini, NIH, Bethesda) and the cells were harvested 40 h after infection. Treatment of the sample was as described in Table 1.

The values presented in the table have been corrected for reagent background, obtained from a similar hybridization carried out without sample.

Example 4

Detection of Bacillus amyloliquefaciens by sandwich hybridization (Table 4)

The reagents were fragments of the α-amylase gens of B. amyloliquefaciens E18 (Technical Research Centre of Finland, VTT), which were isolated for the purpose of this

test from the recombinant plasmid pKTH10 (Palva, I., et al. (1981) Gene, 15 43-51) by treatment with restriction enzyme and subsequent agarose gel electrophoresis. The fragments used for this test were the ClaI-EcoRI fragment (460 base pairs)(ClaI Boehringer Mannheim) and the EcoRI-BamHI fragment (1500 base pairs). The EcoRI-BamHI fragment was attached to the filter and the ClaI-EcoRI fragment was labelled with ^{125}I by nick translation.

As can be seen from Table 4 the B. amyloliquefaciens in a sample was identifiable by sandwich hybridization on the basis of the single α-amylase gene. E. coli gave a negative result in this test (indistinguishable from the background).

Table 4

Bacterial diagnostics by sandwich hybridization

Sample	Filters (cmp)		
	α-amylase 1)	Calf thymus 2)	Blank 3)
pKTH10-plasmid-DNA (linearized) 1 μg	5773	47	-
No sample	-	-	-
E. coli HB101 (10^9)	-	-	-
Bacillus amyloliquefaciens (3 x 10^9)	3377	-	-
Bacillus amyloliquefaciens (10^9)	2871	-	-

Filters:

1) The EcoRI-BamHI fragment of the α-amylase gene from plasmid pKTH10, 0.35 μg
2) Calf thymus DNA, 1 μg
3) Blank (no DNA)

Labelled nucleic acid reagent:

The ClaI-EcoRI fragment of the α-amylase gene from plasmid pKTH10, specific activity 35 x 10^6 cpm/μg (200000 cpm ^{125}I/reaction

Hybridization:

As described in Table 1.

Washing:

As described in Table 1

Samples:

Bacterial samples were treated with lysozyme (67 μg/ml) for 30 min at 37°C; 5 mM EDTA was added to E. coli samples, too. After the treatment SDS was added to all the the samples (final concentration 2 %), which were then passed twice through a fine needle to reduce their viscosity before being denatured by boiling as described in the text relating to handling of samples.

The values appearing in the table have been corrected for reagent background, obtained from a similar hybridization without sample.

Example 5

An example of a reagent combination kit based on the sandwich hybridization method (Table 5)

The samples investigated in this test were cells infected by three viruses (adenovirus, SV40 virus and Herpex simplex virus) and a sample containing Bacillus amyloliquefaciens

bacteria. The following reagents were all simultaneously added to each sample, 5 filters, each containing one type of DNA from SV40 virus, adenovirus, Bacillus amyloliquefaciens α-amylase gene and calf thymus, as well as a filter containing no DNA at al; in addition 200000 cpm of each of the following labelled nucleic acid reagents: SV40 virus-, adenovirus- and α-amylase gene DNA-reagent.

Our example shows that it is possible, without division or dilution of the sample, to investigate simultaneously a suitable series of microbes by adding the reagent combination to the sample. The sample may contain both viral and bacterial nucleic acid. The filters can be recognized by a sign (= mark, tags), which identifies the sequence it contains and tags, which microbe was attached/hybridized to it. The signs can be numbers or letters, e.g. 1 or SV40 2 or Ad etc. or other markers as ✱ for SV40 or Δ for AD or o for Bacillus.

Table 5

A kit based on the filter hybridization technique

Sample	Filters (cpm)				
	SV40 1)	Adeno 2)	α-amylase 3)	Calf thymus 4)	Blank 5)
Cells infected with SV40 virus (10^6)	18390	2	13	22	31
Cells infected with adenovirus type 2 (6×10^5)	-	8750	5	13	-
Cells infected with Herpex simplex virus (10^6)	-	-	-	5	13
Bacillus amyloliquefaciens (10^9)	15	8	6500	16	5
Non-infected cells	-	-	-	-	-

Filters:

1) As in Table 3
2) As in Table 1
3) As in Table 4
4) Calf thymus DNA, 1 µg
5) Blank (no DNA)

Labelled nucleic acid reagents:

SV40 virus as in Table 3
Adenovirus as in Table 1
α-amylase gene as in Table 4

Hybridization:
As in Table 1

Washing:
As in Table 1

Samples:
Cell samples infected with SV40 virus and adenovirus have been described in Tables 3 and 1, respectively. 10^6 Vero cells were infected with Herpes simplex virus type 1. The cells were harvested 20 h post infection as cytopathic effect could be observed. The sample was treated as described for adenovirus infected cells (Table 1).

Bacillus amyloliquefaciens sample:
As in Table 4

The values in the table are corrected for reagent background, obtained by carrying out a similar hybridization without sample.

Example 6

Detection of Escherichia coli by sandwich hybridization (Table 6)

The reagents were prepared from the ompA-gene (outer membrane protein A-gene) of Escherichia coli.

The hybrid plasmids pKTH40 and pKTH45, used as starting material, were prepared from the pTU100 plasmid described by Henning et al. (1979) Proc. Natl. Acad. Sci. USA 76, 4360-4364.

The plasmid pKTH45 (deposited at KTL or National Public Health Institute Helsinki No EH 257), used as a filter reagent, was composed of 740 base pairs from 5' -terminal end of the ompA-gene

inserted into the pBR322-plasmid.

The plasmid pKTH40 contains 300 base pairs from the 3' -terminal end of the ompA-gene and the immediately following 1400 base pairs from the genome of E. coli. The pKTH40 plasmid was cleaved with the BamHI restriction enzyme to receive the DNA fragment of E. coli, which contains the 1700 base pairs mentioned above. This fragment was transferred to the single-stranded bacteriophage M13mp7 according to the methods described by (Messing et al. (1981), Nucl. Acids Res. 9, 309-321, Heidecker et al. (1980), Gene 10, 69-73, Gardner et al. (1981), Nucl. Acids Res. 9, 2871-2888). The recombination-phage mKTH1207 (deposited at KTL no. EH 256) was labelled with ^{125}I-isotope as described on page 9 under the heading "Other labelling methods" and was used as a probe in the sandwich hybridization method.

DNA from disrupted E. coli cells, as well as isolated, purified DNA from E. coli can be detected by sandwich hybridization as shown in table 6.

Table 6

Detection of Escherichia coli by sandwich hybridization

Sample	Filters (cpm)		
	ompA 1)	Calf thymus 2)	Blank 3)
E. coli K12 HB101 DNA a) 2×10^7	282	-	-
E. coli K12 HB101 DNA a) 2×10^8	2206	-	-
E. coli K12 HB101 Cells b) 2×10^7	1113	-	-
E. coli K12 HB101 Cells b) 2×10^8	2327	12	5

a) number of DNA-molecules, b) number of cells

Filters:

1) pKTH45 plasmid 1,088 μg (2×10^{11} molecules)
2) Calf thymus DNA 1,088 μg
3) Blank (no DNA)

Labelled nucleic acid reagents:

mKTH1207, specific activity 8×10^7 cpm/μg DNA (200000 cpm/reaction)

Hybridization:

4 x SSC, 1 x Denhardt solution without BSA (bovine serum albumin), 0,25 % SDS, 200 μg/ml Herring sperm DNA, 17,5 h, +

Washing:

As described in table 1

Samples:

E. coli K12 HB101 - DNA was isolated according to the Marmur-method described by Marmur (1961) J. Mol. Biol. 3, 208-218. DNA was denaturated at 7 mM NaOH, 100°C, 5 min.

The cells were treated with lysozyme (500 µg/ml), EDTA (70 mM +37°C, 30 min), SDS (0,25 %, +65°C) and the free DNA was denaturated by boiling at 14 mM NaOH, +100°C, 5 min).

The values presented in the table have been corrected for reagent background obtained from a similar hybridization without sample.

CLAIMS

1. The use of the technique of sandwich hybridization of nucleic acids, said technique comprising contacting single-stranded nucleic acid from a microorganism with a pair of different nucleic acid reagents, both reagents of the pair being single-stranded and complementary with the microorganism-derived nucleic acid and one of the pair being a nucleic acid fragment attached to a solid carrier whilst the other is a nucleic acid fragment labelled with a marker, whereby a labelled hybrid is formed attached to the solid carrier, for the identification of a microorganism or group of microorganisms present in a sample, the pair of nucleic acid reagents being selected according to the microorganism or group of microorganisms expected to be present in the sample and the correctness of the identification being tested by detection of the extent of formation of a labelled hybrid attached to the solid carrier.

2. The use of the technique of sandwich hybridization of nucleic acid according to claim 1, characterized in that a plurality of the pairs of nucleic acid reagents are used, each pair being complementary with nucleic acid derived from a different microorganism or group of microorganisms, any one of which is expected to be present in the sample.

3. The use of the technique of sandwich hybridization of nucleic acid according to claim 2, characterized in that a plurality of different microorganisms or groups of microorganisms are present in the sample and are detected in one and the same undivided sample by the use of a variety of different pairs of the nucleic acid reagents complementary with nucleic acids from the different microorganisms or groups of microorganisms expected to be present in the sample.

4. A method of identifying microorganisms or groups of microorganisms present in a sample, by means of sandwich hybridization of nucleic acids, using a pair of completely different nucleic acid reagents both complementary to and capable of hybridizing with nucleic acid from the same known microorganism or group of microorganisms, one of the pair of nucleic acid reagents being a single-stranded nucleic acid fragment attached to a solid carrier and the other being a single-stranded nucleic acid fragment labelled with a marker, whereby on successful hybridization a labelled hybrid attached to the solid carrier is formed, the method being characterized in that a plurality of microorganisms or groups of microorganisms present in a single undivided sample are identified by bringing nucleic acids from the microorganisms in the sample, after they have been rendered single-stranded, into contact with a plurality of the pairs of completely different nucleic acid reagents, reagents in different pairs being complementary with and capable of hybridizing with nucleic acid from different known microorganisms or groups of microorganisms, and detecting the formation or non-formation of labelled hybrids attached to a solid carrier in respect of each of the pairs of nucleic acid reagents.

5. A reagent combination for the use claimed in claim 2 or 3 or the method claimed in claim 4, characterized by the presence of a plurality of pairs of different nucleic acid reagents, the two reagents of the same pair in each case being complementary to and capable of hybridizing with nucleic acid from the same microorganism or group of microorganisms and the reagents of different pairs being complementary to and capable of hybridizing with nucleic acid from different microorganisms or groups of microorganisms, one reagent of each pair being a single-stranded nucleic acid fragment attached to a solid carrier and the other reagent of each pair being a single-stranded

nucleic acid fragment labelled with a marker.

6. A reagent combination according to claim 5, characterized in that the two nucleic acid reagents of the same pair are in each case fragments produced either directly from the genome of the microorganism to which they are complementary or by means of a recombinant DNA technique and subsequently rendered single-stranded, and one of the reagents is attached to a solid carrier and the other is labelled with a marker.

7. A reagent combination according to claim 5 or 6, characterized in that one or more of the pairs of nucleic acid reagents is complementary to and capable of hybridizing with nucleic acid from a bacterium or virus associated with a respiratory infection or diarrhoea, a bacterium, virus, yeast or protozoon associated with a venereal disease or a bacterium associated with sepsis or with failures in food hygiene.

8. A reagent combination according to claim 5 or 6, characterized in that one of the pairs of nucleic acid reagents is complementary to and capable of hybridizing with nucleic acid from adenovirus, said pair consisting of the adenovirus recombinant plasmid Ad_2DpBR322 attached to a solid carrier and the adenovirus Ad_2-BamHI C-fragment labelled with a marker.

9. A reagent combination according to any of claims 5 to 8, characterized in that one of the pairs of nucleic acid reagents is complementary to and capable of hybridizing with nucleic acid from Semliki Forest virus, said pair consisting of the EcoRI-XhoI fragment A of the pKTH312 plasmid attached to a solid carrier and the EcoRI-XhoI fragment B of the pKTH312 plasmid labelled with a marker.

10. A reagent combination according to any of claims 5 to 9, characterized in that one of the pairs of nucleic

acid reagents is complementary to and capable of hybridizing with nucleic acid from SV40 virus, said pair consisting of the PstI B fragment of SV40 virus attached to a solid carrier and the PstI A fragment of SV40 virus labelled with a marker.

11. A reagent combination according to any of claims 5 to 10, characterized in that one of the pairs of nucleic acid reagents is complementary to and capable of hybridizing with nucleic acid from Bacillus amyloliquefaciens, said pair consisting of the EcoRI-BamHI fragment of the α-amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 attached to a solid carrier and the ClaI-EcoRI fragmentof the α-amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 labelled with a marker.

12. A reagent combination according to claim 5, characterized in that one of the pairs of nucleic acid reagents is complementary to and capable of hybridizing with nucleic acid from E. coli, said pair consisting of the plasmid pKTH45 attached to a solid carrier and the recombination-phage mKTH1207 labelled with a marker.

European Patent Office

EUROPEAN SEARCH REPORT

Application number 0079139

EP 82305489.5

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 3)
D,A	A.R. DUNN AND J.A. HASSELL, "Cell", vol. 12, no. 1, September 1977 MASSACHUSETTS INSTITUT OF TECHNOLOGY pages 23-36 --	1	C 12 Q 1/68 C 12 Q 1/04
A	GB - A - 2 019 408 (INSTITUT PASTEUR) * Abstract * --	1	
A	US - A - 3 930 956 (E. JUNI) * Abstract * ----	1	

TECHNICAL FIELDS SEARCHED (Int Cl. 3)

C 12 Q

X The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	19-01-1983	SCHNASS

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document
T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons
& : member of the same patent family, corresponding document